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| <b>(54) Title:</b> PLANT GENETIC MANIPULATION<br><br><b>(57) Abstract</b><br><br>"Transit peptides" are known which direct nuclear-encoded chloroplastidic proteins to the chloroplast. Corresponding mitochondrial transit peptides are also known. The invention provides transit peptides which direct nuclear-encoded proteins to both the chloroplast and the mitochondrion. The prototype is derived from glutathione reductase from pea ( <i>Pisum sativum</i> L.). A DNA sequence encoding a fusion protein of the transit peptide fused to a protein of interest can be used to generate transgenic plants.   |           |   |

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PLANT GENETIC MANIPULATION

This invention relates to plant molecular biology. In particular, it relates to the targeting of plant organelles by chimeric preproteins.

The N-terminal pre-sequences of certain plant proteins are known to be able to target the protein either to the chloroplast or to the mitochondrion, with varying degrees of efficiency. These pre-sequences, which are known as 'transit peptides' or 'targeting sequences', are necessary for nuclear-encoded chloroplastidic or mitochondrial proteins and are generally cleaved from the mature protein during or after translocation into the organelle. Examples of transit peptides known to be chloroplast-specific include N-terminal pre-sequences derived from:

the small subunit of ribulose-1,5-bisphosphate carboxylase, otherwise known as rubisco (Schreier et al., *The EMBO Journal* 4(1) 25-32 (1985), Van den Broeck et al. *Nature* 313 358-363 (1985) and Guerineau et al. *Nucleic Acids Research* 16(23) 11380 (1988));

the chlorophyll a/b binding proteins (Kavanagh et al., *Mol. Gen. Genet* 215 38-45 (1988)); and

glutathione reductase (Creissen et al. *The Plant Journal* 2(1) 129-131 (1991)).

Examples of transit peptides known to be mitochondrion-specific include N-terminal pre-sequences derived from:

the  $\beta$ -subunit of mitochondrial ATP synthase (Boutry et al., *Nature* 328 340-342 (1987)); and

5 mitochondrial tryptophanyl-tRNA synthetase (Schmitz & Lonsdale *The Plant Cell* 1 783-791 (1989)).

By the use of recombinant DNA encoding proteins which are a fusion between one of the above transit peptides and a protein of interest it is possible to target the protein  
10 of interest to the chloroplast or mitochondrion. However, it is generally accepted in the art, as represented by the literature highlighted above, that transit peptides are specific for one or other organelle. Now there are a number of situations in which it is  
15 desirable for (often heterologous) proteins of interest to be targeted to both chloroplasts and mitochondria, whether for the purpose of manipulating metabolism in both organelles or for other reasons. Examples include the manipulation of the antioxidant, enzymic or other  
20 content of these organelles to increase tolerance to abiotic or environmental stress and the enhancement of resistance to herbicides which interfere with electron transport in both organelles.

25 At present, the only way in which it is apparent from the art that both organelles can be targeted is to transform the plant with recombinant DNA encoding two fusion proteins: one comprising the protein of interest fused to a chloroplast-specific transit peptide and the other  
30 comprising the protein fused to a mitochondrion-specific transit peptide. The plant therefore needs to be made doubly transgenic. This clearly involves significant effort in terms of the initial transformation work and there is always the problem that the two transgenes may

not co-segregate in progeny of the plant, making subsequent breeding from a transgenic parent difficult. Regulatory issues may also militate against doubly transgenic plants.

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There is therefore a real need in the art for a transit peptide which co-targets to both the chloroplast and the mitochondrion, to enable a plant to be made singly transgenic for a transgene which encodes a protein which is targeted to both organelles. The present invention is based on the remarkable discovery that one of the transit peptides which was thought to be specific for the chloroplast does in fact meet this need. The transit peptide in question is the glutathione reductase pre-sequence. Neither the Creissen et al. paper *supra* nor, it is believed, the rest of the literature actually discloses fusions between the glutathione reductase pre-sequence and a heterologous protein.

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According to a first aspect of the invention, there is provided a fusion protein comprising a protein of interest and sufficient of the N-terminal pre-sequence of a glutathione reductase to cause targeting of the protein to both chloroplasts and mitochondria.

The glutathione reductase (GR) which supplies the pre-sequence may be derived from any suitable organism. All that is required is that the organism in question be such that its GR has the ability to co-target both chloroplasts and mitochondria in plants. The GR will usually be derived from a plant, particularly a higher plant such as those of the class *Gymnospermae* or, preferably, *Angiospermae*. Angiosperms of the family *Leguminosae* are preferred, particularly species of the

genus *Pisum*. A highly suitable source of GR is the pea (*Pisum sativum* L.).

5 It is not necessary for all the natural GR pre-sequence, from whatever source, to be present: only the minimum amount necessary to achieve the targeting (and, if desired, to enable subsequent cleavage) has to be present.

10 In the case of pea GR, the complete pre-GR sequence is set out in Creissen et.al. supra and the transit peptide includes at least some of the following residues:

15 MNQAMATPLS LSCCSPTLTR STLFFTKTFP FSRSFSTPLP  
LSTKTLISLS PPHRTFAVRA ESQNGADPAR Q

20 The natural transit peptide comprises about 60 to 70 residues, all of which may be present in embodiments of the present invention.

It is preferred that at least the following residues be present:

25 MNQAMATPLS LSCCSPTLTR STLFFTKTFP FSRSFSTPLP  
LSTKTLISLS PPHRT

30 as those residues are not shared with *Ps. aeruginosa*, *E. coli* or human glutathione reductase. The residues FAV may also be present to the C-terminal side of that sequence; and the further residues RAESQNGADPARQ may be further added to the C-terminal side.

35 While it is expected with the current state of the art that it will be preferred to use transit peptides in the

invention which are identical to natural GR transit peptides (particularly that of *P. sativum*), a degree of divergence from the natural or consensus sequence can be tolerated in the invention provided only that the co-targeting ability of the transit peptide is not lost. Typically, a mutant, variant or derivative transit peptide useful in the invention will be homologous with the natural sequence to the extent of about 60% or even 90% or 95%.

The invention is not limited by the protein of interest which may be targeted to both chloroplasts and mitochondria by means of the invention. Some proteins of interest will be enzymes (even mature glutathione reductase), but whatever their nature their presence will simply be dictated by the particular purpose of the embodiment of the invention in question. Among the preferred purposes of embodiments of the invention are:

1. The manipulation of the antioxidant content of these organelles (eg, glutathione, ascorbate) in order to enhance tolerance to a range of abiotic stresses, thus protecting both major sites of oxygen metabolism in the plant cell (Creissen et al, *Proc. Royal Society Edinburgh* (1994));

2. The simultaneous targeting to both compartments of enzymes involved in oxyradical scavenging or antioxidant metabolism e.g. pea Cu/Zn superoxidase (EMBL accession code: PSSOD), pea Mn superoxide dismutase (EMBL accession code: PSSVPOXRE), maize catalase (EMBL accession code: ZMCATZ), pea ascorbate peroxidase (EMBL accession code: PSAPXIA), *E. coli* glutathione reductase (EMBL accession code:

5 ECSGSHII) and *E. coli* gamma glutanyl cysteine synthetase (EMBL accession code: ECGSHI) for increased scavenging of superoxide leading to enhanced tolerance to environmental stress (see, for example, Bowler et al., *The EMBO Journal* 8(1) 31-38 (1989)); and

10 3. To enhance tolerance to herbicides which disrupt a critical biochemical process e.g. electron transport in both chloroplasts and mitochondria, by co-targeting resistance genes to these herbicides; for example, resistance to paraquat or acifluorfen.

15 Fusion proteins of the invention may in principle be made by any convenient process, including *de novo* chemical synthesis. In practice, recombinant DNA technology provides the method of choice, and the fusion proteins will be expressed from a recombinant DNA molecule.

20 According to a second aspect of the invention, there is provided a recombinant DNA molecule encoding a fusion of a protein of interest with sufficient of the N-terminal pre-sequence of a glutathione reductase to cause targeting of the protein to both chloroplasts and  
25 mitochondria.

30 Recombinant or isolated DNA molecules encoding the transit peptide alone, in the absence of the mature GR protein-coding sequence, are useful for ligation to DNA sequences encoding proteins of interest. According to a third aspect of the invention, therefore, there is provided a recombinant or isolated DNA molecule encoding sufficient of the N-terminal pre-sequence of a glutathione reductase to cause targeting of a protein to



both chloroplasts and mitochondria, provided that in the said isolated or recombinant DNA molecule the DNA encoding the pre-sequence is not precisely fused to DNA encoding mature glutathione reductase.

5

DNA molecules in accordance with the invention may, if encoding a natural GR transit peptide, correspond to a cDNA or genomic sequence; in other words the presence or absence of any natural introns is not critical to the functioning of the invention, although it may be expected that the presence of one or more natural introns can have implications for expression efficiency.

Recombinant DNA in accordance with the invention may be in the form of a vector. The vector may for example be a plasmid, cosmid or phage. Vectors will frequently include one or more selectable markers to enable selection of cells transfected (or transformed: the terms are used interchangeably in this specification) with them and, preferably, to enable selection of cells harbouring vectors incorporating heterologous DNA. Appropriate start and stop signals will generally be present. Additionally, if the vector is intended for expression, sufficient regulatory sequences to drive expression will be present; however, DNA in accordance with the invention will generally be expressed in cells containing both chloroplasts and mitochondria, e.g. plant cells and algae. Vectors not including microbial regulatory sequences are useful as cloning vectors. For expression in plants, a plant promoter will generally be present operably coupled to sequences to be expressed; any suitable promoter may be used, such as, for example, the 35S Cauliflower Mosaic Virus (CaMV) promoter, the rubisco small subunit (rbs c), a ubiquitin, the plastocyanin or

the *Agrobacterium* nopaline synthase (nos) promoter.

Cloning vectors can be introduced into *E. coli* or another suitable host which facilitate their manipulation.

5 According to a fourth aspect of the invention, there is therefore provided a host cell transfected or transformed with DNA as described above.

10 DNA in accordance with the invention can be prepared by any convenient method involving coupling together successive nucleotides, and/or ligating oligo- and/or poly-nucleotides, including *in vitro* processes, but again recombinant DNA technology forms the method of choice.

15 Ultimately, DNA in accordance with the invention will be introduced into plant cells, by any suitable means. According to a fifth aspect of the invention, there is provided a plant cell including DNA in accordance with the invention as described above.

20 Plants transformed with the DNA segment containing the pre-sequence may be produced by standard techniques which are already known for the genetic manipulation of plants. DNA can be transformed into plant cells using any  
25 suitable technology, such as a disarmed Ti-plasmid vector carried by *Agrobacterium* exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, Bevan, *Nucleic Acids Research*, 12(22): 8711-8721 (1984)).  
particle or microprojectile bombardment (US-A-5100792, EP-A-444882, EP-A-434616), microinjection (WO 92/09696, 30 WO 94/00583, EP-A-331083, EP-A-175966), electroporation (EP-A-290395, WO-A-8706614). *Agrobacterium* transformation is widely used by those skilled in the art to transform dicotyledonous species. Although

*Agrobacterium* has been reported to be able to transform foreign DNA into some monocotyledonous species (WO 92/14828), microprojectile bombardments, electroporation and direct uptake are preferred where *Agrobacterium* is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, e.g. bombardment with *Agrobacterium* coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with *Agrobacterium* (EP-A-486233).

The particular choice of transformation technology will be determined by its efficiency to transform certain plant species, as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce chimeric genes into the plant cells or algae is not essential to the invention.

Alternatively, the foreign DNA could be introduced directly into plant cells using a particle bombardment apparatus. This method is preferred where *Agrobacterium* is ineffective, for example where the recipient plant is monocotyledonous. Any other method that provides for the stable incorporation of the DNA within the nuclear DNA of any plant cell of any species would also be suitable.

30

DNA in accordance with some embodiments of the invention may also contain a second chimeric gene (a "marker" gene) that enables a transformed plant containing the foreign DNA to be easily distinguished from other plants that do

not contain the foreign DNA. Examples of such a marker gene include antibiotic resistance (Herrera-Estrella et al, *The EMBO Journal*, 2 987-995 (1983)), herbicide resistance (EP-A-0242246) and glucuronidase (GUS) expression (EP-A-0344029); however, in some embodiments of the invention the protein of interest may serve as its own marker gene and so no second marker will necessarily be needed. Expression of the marker gene, if present, is preferably controlled by a second promoter (which may also be the 35S CaMV promoter). However any other suitable second promoter could be used.

A whole plant can be regenerated from a single transformed plant cell, and the invention therefore provides in a sixth aspect transgenic plants (or parts of them, such as propagating material) including DNA in accordance with the invention as described above. The regeneration can proceed by known methods.

A singular advantage, quite literally, of the invention is that, as discussed above, a plant does not have to be doubly transgenic if the same protein of interest is to be targeted to both mitochondria and chloroplasts. According to a seventh aspect of the invention, therefore, there is provided a plant having a transgene encoding a fusion of a protein and sufficient of the N-terminal leader sequence of a plant glutathione reductase to cause targeting of the said protein to both mitochondria and chloroplasts, wherein the plant does not have a further transgene which encodes a second mitochondrion- or chloroplast-targeting sequence fused to the said protein.

Transgenic plants in accordance with the invention are

not limited by species. Much work on transgenic plants has been done in tobacco (*Nicotiana tabacum*), which is consequently one of the better understood transgenic hosts (and which is represented in the examples of this invention, shown below). However, the invention is in no sense limited in its usefulness to tobacco or any other individual species.

In general from the above discussions it can be seen that the invention also provides, in an eighth aspect, a method of targeting a protein to both mitochondria and chloroplasts, the method comprising expressing the said protein in a plant as a fusion with sufficient of the N-terminal leader sequence of a plant glutathione reductase to cause targeting of the protein to both organelles.

Preferred features of each aspect of the invention are as for each of the other aspects, *mutatis mutandis*.

The invention will now be illustrated by the following examples. The examples refer to the accompanying drawings, in which:

FIGURE 1 is a map of plasmid pGR46; 'LB' and 'RB' represent the left and right borders, respectively;

FIGURE 2 shows the complete cDNA sequence of pea glutathione reductase, with the deduced amino acid sequence;

FIGURE 3 shows an amino acid alignment of glutathione reductases from pea (Peagr), *Pseudomonas aeruginosa* (Psagr), *Escherichia coli* (Ecgr) and man (Humgr); conserved regions are shaded;

FIGURE 4 is a map of plasmid pGR50; 'LB' and 'RB' represent the left and right borders, respectively; and

5           FIGURE 5 is a map of plasmid pGR42.

EXAMPLE 1 - Isolation of Glutathione Reductase cDNAs

10           Glutathione reductase (GR) CDNAS were isolated from a bacteriophage  $\lambda$ gt11 cDNA expression library constructed from poly(A)<sup>+</sup> RNA isolated from 14 day old pea seedlings.  $\lambda$ gt11 is available from Amersham International plc, Amersham and the cDNA library was constructed essentially following the supplier's instructions and as indicated by

15           Creissen et al. (*The Plant Journal* 2(1) 129-131 (1992)). The appropriate recombinant phage were identified by immunodetection using an anti-GR antiserum raised in rabbits against purified pea GR protein (Edwards et al., *Planta* 180 278-284 (1990)). Immunodetection was achieved

20           by virtue of the recognition by anti-GR of antibodies of the phage-directed synthesis of a  $\beta$ -galactosidase-GR fusion protein. After purification of candidate phage and isolation of their DNA, cDNA inserts were subcloned into the plasmid vector pBLUESCRIPT SKII+ (Stratagene Ltd.,

25           Cambridge) as *Bam*HI fragments. DNA sequence analysis was performed using the dideoxy-chain termination procedure (Sanger et al., *Proc. Nat'l. Acad. Sci. USA* 74 5463-5467 (1977)) on single-stranded and double-stranded DNA templates. The largest cDNA recovered at this stage was

30           termed pGR27 which appeared to encode the mature GR peptide. The cDNA pGR201 was subsequently recovered from the same cDNA library using the 5'-region (co-ordinate 254-393 of the published sequence) of pGR27 as a radiolabelled probe. DNA sequence analysis of the

subcloned cDNA in pGR201 revealed a coding sequence which was clearly identified as GR by amino-acid sequence homology to known GR sequences from other sources (*Homo sapiens*, *Escherichia coli* and *Pseudomonas aeruginosa*).  
5 However the pGR201-encoded cDNA also encodes an N-terminal extension, which at the time of first analysis, was determined to be most likely a chloroplast targeting sequence (Creissen *et al*, *supra*). Upstream of the first in-frame methionine initiator codon (AUG) was a  
10 translational stop codon. Therefore the cDNA in pGR201 was deemed to encode the full length GR preprotein.

EXAMPLE 2 - Construction of chimeric genes; in vitro manipulation of pGR201

15

As part of a programme for engineering abiotic stress tolerance in plants, GR cDNAs were manipulated *in vitro* to produce the following chimeric genes.

20

pGR42 was constructed as follows: The *EcoRV*-*Bam*HI fragment (co-ordinates 18-2029) from pGR201 was recovered and inserted into the expression cassette pJIT163-*Bgl*II. pJIT163-*Bgl*II contains 35S promoter and polyadenylation sequences from cauliflower mosaic virus (CaMV) separated  
25 by a restriction-site polylinker. pJIT163-*Bgl*II was made from pJIT163 (Guerineau *et al*, *Plant Molecular Biology*, 18 815-818 (1992)) by insertion of a *Bgl*II linker (5'-GCAGATCTCC-3') into the *Sac*I site of pJIT163 located at the 5'-end of the 35S promoter.

30

EXAMPLE 3 - Insertion of chimeric GR gene into a binary Ti vector for Agrobacterium-mediated transformation of plants

5 The chimeric gene of Example 2 above was subcloned as a BglII fragment into the BamHI site of pBinLuc23. pBinLuc23 comprises the binary Ti vector pBin19 (Bevan, *Nucleic Acids Research*, 12 8711-8721 (1984)) into which a 35S promoter-luciferase gene (Mullineaux et al.,  
10 *Nucleic Acids Research*, 18 7259-7265 (1990)) was inserted at the SacI site. The chimeric GR gene was inserted between a Kanamycin resistance gene and the 35S-luciferase gene within the T-DNA borders of the vector. The plasmid was designated pGR46 (Figure 1). The plasmid  
15 pGR46 was introduced into *Agrobacterium tumefaciens* strain LBA4404 by a triparental mating technique (Ditta et al, *Proc. Natl. Acad. Sci. USA*, 77: 7374 (1990)). The *Agrobacterium* containing pGR46 was used to transform tobacco (*Nicotiana tabacum* L. cv. Samsun NN).

20

EXAMPLE 4 - Transformation of tobacco

Leaf discs of cv. Samsun NN were co-cultivated with *A. tumefaciens* containing the pGR46 essentially as described  
25 by Guerineau et al., *Plant Molecular Biology*, 15 127-136 (1990). Potentially transformed shoots were identified as being resistant to 100 mg/l kanamycin sulphate in the growth medium. Putative transformed shoots were rooted on 100 mg/l kanamycin sulphate-containing rooting medium,  
30 and confirmed by screening the shoots for luciferase activity as described by Mullineaux et al. (1990) supra. Kanamycin resistant, luciferase positive (Kan<sup>r</sup>, luc<sup>+</sup>) plantlets were potted in soil and grown to maturity in the glasshouse. Seed was collected from self-pollinated



plants ( $T_1$  progeny).

EXAMPLE 5 - Compartmentation analysis

5  $T_1$  progeny from 5 independently transformed pGR46 lines were germinated and screened for segregation of luciferase activity (ie  $luc^+luc^-$ ). GR activity was assayed (Edwards et al., *Planta*, 180 278-284 (1990)) from total pooled extracts of each line of  $luc^+$  seedlings was  
10 determined (Table 1).

Table 1

| LINE            | GR ACTIVITY (nmols<br>NADPH oxidised $\text{min}^{-1}$<br>mg protein) | INCREASE OVER<br>CONTROL (fold) |
|-----------------|---|---------------------------------|
| control tobacco | 6   | 1                               |
| 46-19           | 102   | 17                              |
| 46-20           | 205   | 34                              |
| 46-23           | 88  | 15                              |
| 46-27           | 124   | 21                              |
| 46-29           | 123   | 20                              |

15 Two independently transformed lines were selected for further analysis. These were 46-23 and 46-27. Enhanced synthesis of GR was determined by immunodetection on Western blots.  $Luc^+$  and  $luc^-$   $T_1$  plants from each of these  
20 lines were used as sources of chloroplast and mitochondria.

Chloroplast fractionation was carried out using the method of Boutry et al., *Nature*, 328 340-342 (1987) with  
30 the following modifications:

1. The plant material was homogenised with a Polytron.
2. 1 mM EDTA was added to the grinding medium.
3. Percoll was diluted in the grinding medium, rather

than second suspension medium.

4. After recovery of purified chloroplast fraction from Percoll gradients, the chloroplasts were washed in 10 volumes of HEPES-sorbitol medium (50 mM HEPES, 330 mM sorbitol, 10 mM NaCl, 1 mM MgCl<sub>2</sub>, 2mM KH<sub>2</sub>PO<sub>4</sub>, pH7.6) and resuspended in the same medium.
5. Chloroplasts were lysed for enzyme assays by adding an equal volume of lysis buffer (50 mM Tris-HCl pH7.5, 5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 1 mM EDTA, 0.2% Triton X-100).

To confirm the purity of chloroplast fractions, enzymes were assayed which are specific to each of the major cell compartments (Edwards et al., *Planta*, 180 278-284 (1990)). These are glyceraldehyde 3-phosphate dehydrogenase (GAPDH; chloroplast; Wu and Racker, *J. Biological Chemistry*, 234 1029-1035 (1959)); cytochrome C oxidase (CCO; mitochondria; Tolbert, *Methods of Enzymology*, 31A 734-746 (1974)) and pyrophosphate-dependent phosphofructokinase (PPi-PFK; cytosol; Journet and Douce, *Plant Physiology*, 79 458-467 (1985)). Data from two experiments, one on each of the selected lines, are presented in Table 2.

Table 2

| LINE/FRACTION                         | GR  | GAPDH | CCO | PPi-PFK |
|---------------------------------------|-----|-------|-----|---------|
| 46-27, luc <sup>+</sup> : total       | 220 | 80    | N/A | 40      |
| 46-27, luc <sup>-</sup> : total       | 10  | 50    | N/A | 20      |
| 46-27, luc <sup>+</sup> : total       | 100 | 20    | N/A | 20      |
| 46-23, luc <sup>+</sup> : total       | 20  | 30    | N/A | 30      |
| 46-27, luc <sup>+</sup> : chloroplast | 130 | 30    | 0   | 0       |
| 46-27, luc <sup>-</sup> : chloroplast | 8   | 20    | 0   | 0       |
| 46-23, luc <sup>+</sup> : chloroplast | 70  | 30    | 0   | 0       |
| 46-23, luc <sup>-</sup> : chloroplast | 10  | 30    | 0   | 0       |

NOTES One unit of each of GR and GAPDH activity represents one nmol NADPH oxidised per minute per mg protein. One unit of CCO activity represents one  $\mu$ mol reduced cytochrome C oxidised per minute per mg protein. One unit of PPi-PFK activity represents one nmol NADH oxidised per minute per mg protein. 'N/A' means not assayed.

The data clearly show the elevation of GR activities in chloroplasts of luc<sup>+</sup> plants compared with their luc<sup>-</sup> siblings, confirming that the product of the pGR201 cDNA is targeted to the chloroplast. Mitochondrion fractionation was carried out using the method of Boutry et al (1987) *supra* as described for chloroplasts with the following modifications:

1. Ascorbic acid was omitted from the grinding medium; and
2. Mitochondria were layered onto a single concentration of Percoll (50% v/v) in grinding medium.

Other than these two specific modifications for mitochondria, all other modifications to the method of Boutry et al, as described for chloroplasts, are used for

the mitochondrion preparation.

The marker enzymes used to establish the purity of the mitochondrion preparation were the same as those used for the chloroplast preparation. Data from two experiments, one on each of the selected lines, are presented in Table 3.

Table 3

| LINE/FRACTION              | GR    | GAPDH | CCO  | PPi-PFK |
|----------------------------|-------|-------|------|---------|
| 46-27, luc': total         | 100   | 80    | 27   | 20      |
| 46-27, luc': total         | 20    | 120   | 24   | 20      |
| 46-23, luc': total         | 90    | 70    | 23   | 10      |
| 46-23, luc': total         | 20    | 90    | 14   | 10      |
| 46-27, luc': mitochondrion | 50    | 0     | 1895 | 0       |
| 46-27, luc': mitochondrion | 0     | 0     | 1895 | 0       |
| 46-23, luc': mitochondrion | 80(*) | 20    | 345  | 0       |
| 46-23, luc': mitochondrion | 0     | 10    | 403  | 0       |

NOTES One unit of each of GR and GAPDH activity represents one nmol NADPH oxidised per minute per mg protein. One unit of CCO activity represents one  $\mu$ mol reduced cytochrome C oxidised per minute per mg protein. One unit of PPi-PFK activity represents one nmol NADH oxidised per minute per mg protein. (\*) = 3% of this GR activity is attributable to chloroplast contamination as indicated by the presence of GAPDH activity in the mitochondrial fraction.

The data clearly show the elevation of GR activities in mitochondria of luc' plants compared with their luc' siblings, showing that the product of the pGR201 cDNA is also targeted to the mitochondrion.

Therefore, taking the data presented in Tables 2 and 3 together, it is clear that the cDNA pGR201 can direct GR to both of these organelles.

EXAMPLE 6 - Construction of a chimeric gene consisting of a fusion between the sequences encoding the amino terminus of pea glutathione reductase and the coding sequence for phosphinothricin acetyl transferase from *Streptomyces hygroscopicus*

**Starting materials:**

The source of the glutathione reductase (GR) sequence was the plasmid pGR42 (see Example 2). This contains the pea glutathione reductase cDNA clone (Creissen et al, 1992, supra) under the control of the CaMV 35S promoter and polyadenylation sequences.

The phosphinothricin acetyl transferase (pat) coding sequence was obtained from the plasmid pIJ4102. pIJ4102 is identical to plasmid pIJ4104 described by White et al, Nucl. Acids Res., 18: 1063 (1990).

**Description of the chimeric GR:pat gene construct:**

The chimeric gene construct comprised the CaMV 35S promoter with duplicated enhancer region and CaMV polyadenylation signals (Guerineau et al, 1992, supra), flanking a fusion between the 5'-region of the pea GR cDNA (pGR201; co-ordinates 18-392) and the pat coding sequence such that translation would be initiated at one of the GR AUG codons and continue to the translational stop codon at the 3'-end of the pat coding sequence. The pat coding sequence data is lodged with the EMBL database as entries SHBRPA and X17220.

**Cloning Strategy:**

1. The pat coding sequence was released from pIJ4102 by digestion with XhoI; followed by treatment with bacteriophage T4 DNA polymerase and subsequent digestion with BglII. The ca. 550bp pat coding sequence was eluted from an agarose gel.
2. The plasmid pGR42 was digested with SacI, followed by treatment with bacteriophage T4 DNA polymerase and subsequent digestion with BamHI. The fragment comprising the vector plus CaMV promoter and polyadenylation sequences and 5' end of GR was eluted from an agarose gel.
3. The chimeric expression cassette pGR48 was generated by ligation of the two fragments from 1 and 2 above.
4. Finally, the chimeric gene was excised as a BglII fragment and ligated into the unique BamHI site of the binary vector pBINLUC23 (see Example 3) to create pGR50.

**Transformation of tobacco:**

The plasmid pGR50 was mobilised into *Agrobacterium tumefaciens* LBA4404 by the triparental mating procedure (Ditta et al, *Proc. Natl. Acad. Sci. USA.* 77: 7374 (1990)) and used to transform tobacco by leaf disc co-cultivation (Horsch et al, *Science*, 223: 496 (1984)). Putative transgenic plants were identified by their ability to root on kanamycin-containing medium. Kanamycin-resistant shoots, which were found also to be expressing the firefly luciferase (T-DNA right border

marker), were transferred to the glasshouse and seeds were collected from self-pollinated plants.

5       Seeds were sown on phosphinothricin-containing medium (10 ug/ml) and were found to exhibit the predicted 3:1 segregation of resistance, confirming that there was a single locus for the T-DNA and that the fusion protein expressed from the chimeric gene was biologically active. The presence of the PAT protein in chloroplasts and  
10       mitochondria are confirmed using the methods detailed above (see Example 5).

CLAIMS

1. A fusion protein comprising a protein of interest  
and sufficient of the N-terminal pre-sequence of a  
glutathione reductase to cause targeting of the protein  
to both chloroplasts and mitochondria.

2. A fusion protein as claimed in claim 1, wherein the  
source of glutathione reductase is the pea (*Pisum sativum*  
L.).

3. A fusion protein as claimed in claim 1 or 2, wherein  
the pre-sequence includes at least some of the following  
residues:

MNQAMATPLS LSCCSPTLTR STLFFTKTFP FSRSFSTPLP  
LSTKTLISLS PPHRTFAVRA ESQNGADPAR Q.

4. A fusion protein as claimed in claim 3, including at  
least the following residues:

MNQAMATPLS LSCCSPTLTR STLFFTKTFP FSRSFSTPLP  
LSTKTLISLS PPHRT.

5. A fusion protein as claimed in claim 4, further  
including the residues FAV to the C-terminal side of the  
N-terminal pre-sequence.

6. A fusion protein as claimed in claim 5, further  
including the residues RAESQNGADPARQ to the C-terminal  
side of the N-terminal pre-sequence.

7. A fusion protein as claimed in any one of claims 1



to 6, wherein the protein of interest is implicated in the manipulation of the antioxidant content.

5 8. A fusion protein as claimed in any one of claims 1 to 6, wherein the protein of interest is an enzyme involved in oxyradical scavenging or antioxidant metabolism.

10 9. A fusion protein as claimed in claim 8 wherein the protein of interest is superoxide dismutase.

15 10. A fusion protein as claimed in any one of claims 1 to 6, wherein the protein of interest is implicated in the enhancement of tolerance to herbicides which disrupt a critical biochemical process in either chloroplasts or mitochondria or both.

11. A fusion protein as claimed in claim 10 wherein the critical biochemical process is electron transport.

20 12. A recombinant DNA molecule encoding a fusion of a protein of interest with sufficient of the N-terminal pre-sequence of a glutathione reductase to cause targeting of the protein to both chloroplasts and mitochondria.

25 13. A DNA molecule as claimed in claim 12 which encodes a fusion protein as claimed in any one of claims 2 to 11.

30 14. A recombinant or isolated DNA molecule encoding sufficient of the N-terminal pre-sequence of a glutathione reductase to cause targeting of a protein to both chloroplasts and mitochondria, provided that in the said isolated or recombinant DNA molecule the DNA encoding the pre-sequence is not precisely fused to DNA

encoding mature glutathione reductase.

15. DNA as claimed in claim 12, 13 or 14, which is in the form of a vector.

5

16. DNA as claimed in any one of claims 12 to 15, including a functional promoter, operative in a plant.

10

17. DNA as claimed in claim 16, wherein the promoter is the 35S Cauliflower Mosaic Virus (CaMV) promoter.

18. A host cell transfected or transformed with DNA as claimed in any one of claims 12 to 17.

15

19. A plant cell including DNA as claimed in any one of claims 12 to 17.

20. A transgenic plant (or parts of a transgenic plant) including DNA as claimed in any one of claims 12 to 17.

20

21. A plant having a transgene encoding a fusion of a protein and sufficient of the N-terminal leader sequence of a plant glutathione reductase to cause targeting of the said protein to both mitochondria and chloroplasts, wherein the plant does not have a further transgene which encodes a second mitochondrion- or chloroplast-targeting sequence fused to the said protein.

25

22. A method of targeting a protein to both mitochondria and chloroplasts, the method comprising expressing the said protein in a plant as a fusion with sufficient of the N-terminal leader sequence of a plant glutathione reductase to cause targeting of the protein to both organelles.

30

FIG. 1

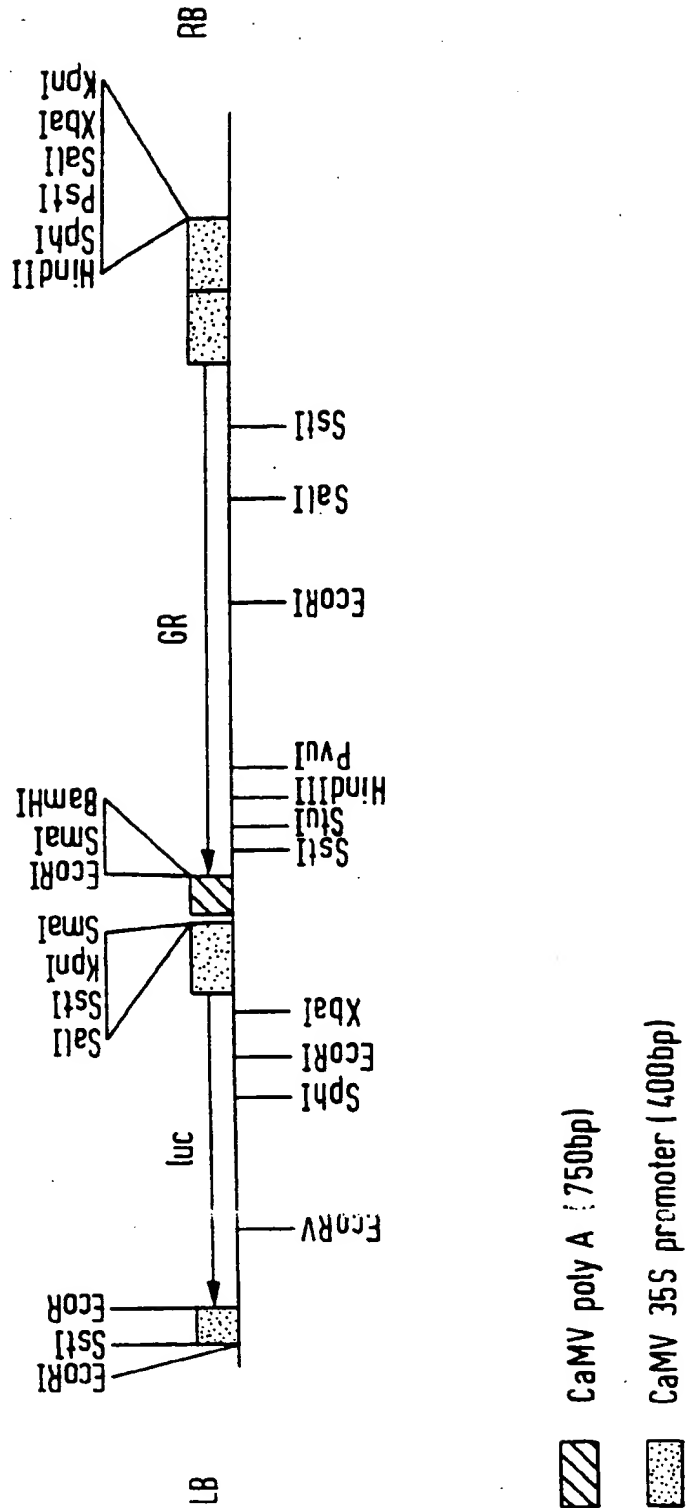


FIGURE 2

1 CTTGTATTACTCCACCGAGATATCCGGATTATCAAGGTGGTTGGTTACTAAGCACCACCG  
L V L L H R D I R I I K V V G Y \* A P P -

61 GGAACAGAGAGGGAAACTCTAATGAACCAAGCAATGGCTACTCCACTTTCTCTCTTGT  
G T E R E T L M N Q A M A T P L S L S C -

121TGTTCTCCAACCTCTAACTCGCAGCACTCTCTTCTTCACCAAAACCTTTCCATTTTCTCGC  
C S P T L T R S T L F F T K T F P F S R -

181TCCTTCTCTACACCTCTCCCTCTCTCCACCAAAACCCTAATTTCCCTCTCTCCACCCCAT  
S F S T P L P L S T K T L I S L S P P H -

241CGCACCTTCGCCGTCGCGCTGAATCCCAAAACGGCGCCGACCCCGCCCGCCAGTATGAC  
R T F A V R A E S Q N G A D P A R Q Y D -

301ITCGACCTTTTTCACCATCGGCGCTGGAAGCGGAGGCGTCCGTGCTTCCCGCTTTGCCTCC  
F D L F T I G A G S G G V R A S R F A S -

361AATTTTCGGCGCTTCTTCTGCTGTCTGCGAGCTCCCTTTCTCTACTATCTCCTCCGATACC  
N F G A S S A V C E L P F S T I S S D T -

421ACCGGTGGTGTGCGCGGCACCTGTGTAATACGGGGATGTGTCCCTAAGAAATTGCTAGTC  
T G G V G G T C V I R G C V P K K L L V -

481TATGCCTCAAAATTCTCTCATGAATTTGAAGAAAGCAATGGTTTTGGATGGAGATATGAC  
Y A S K F S H E F E E S N G F G W R Y D -

541AGTGAACCTAAGCATGACTGGAGTAGTTTGATTGCTAATAAAAACGCCGAGTTGCAGCGG  
S E P K H D W S S L I A N K N A E L Q R -

601CTTACTGGTATCTATAAGAATACTTTGAAAAATGCCGGTGTTAAGTTGATTGAAGGCCGT  
L T G I Y K N T L K N A G V K L I E G R -

661GGAAAGATTGTAGATGCTCACACAGTTGATGTTGATGGGAAGTTATATTCAGCGAAACAC  
G K I V D A H T V D V D G K L Y S A K H -

721ATTTTAGTTTTAGTTGGAGGTGCGACCCTTCATTCCTGATATTCCTGGAAAGGAATATGCA  
I L V S V G G R P F I P D I P G K E Y A -

781ATAGATTGAGATGCTGCCCTTGATTTACCATCAAAGCCTCAGAAGATAGCTATTGTTGGT  
I D S D A A L D L P S K P Q K I A I V G -

841GGGGGTTACATTGCCTTGGAGTTTGCTGGTATCTTTAATGGTTTGAAAAGTGAAGTTTCAT  
G G Y I A L E F A G I F N G L K S E V H -

901GTATTTTATAAGACAAAAGAAGGTTTTGCGGGGATTTGATGAAGAGATTAGAGATTTTGTT  
V F I R Q K K V L R G F D E E I R D F V -

961GCAGAAAATATGGCTCTGAGAGGTATTGAATTCATACTGAGGAGTCTCCTGTAGCTATC  
A E N M A L R G I E F H T E E S P V A I -

1021 ACTAAGGCAGCTGATGGTTCGCTCTCTTTAAAGACCAACAAAGGTAAGGAGGTTTC  
T K A A D G S L S L K T N K G T E E G F -

FIGURE 2 (Cont.)

1081 TCTCATATTATGTTTGCCACTGGACGCTCACCTAATACTAAGGATTGCGCCTGGAGTCT  
S H I M F A T G R S P N T K D L G L E S -

1141 GTTGGTGTGAAAGTGGCTAAAGATGGATCAATAGAGGTTGATGAATACTCTCAAACATCG  
V G V K V A K D G S I E V D E Y S Q T S -

1201 GTTCCTTCTATTTGGGCAATTGGAGATGCTACAAATAGAGTAAATCTCACTCCAGTTGCT  
V P S I W A I G D A T N R V N L T P V A -

1261 TTGATGGAGGGAGTGGCATTAGCAAAAACCTTTGTTTCAGAATGAGCCGACAAAACCTGAT  
L M E G V A L A K T L F Q N E P T K P D -

1321 TATAGGGCTATACCTTCTGCTGTGTTTTCCCAACCACCAATTGGAGGAGTTGGTCTTACA  
Y R A I P S A V F S Q P P I G G V G L T -

1381 GAGGAACAGGCTGCTGAACAATATGGTGATATTGACGTTTTACAGCAAATTTTAGGCCG  
E E Q A A E Q Y G D I D V F T A N F R P -

1441 ATGAAGGCCACCCTCTCTGGGCTTCCAGACCGGGTTTTATGAACTAATAGTCTCTGCA  
M K A T L S G L P D R V F M K L I V S A -

1501 GAAACAAATGTTGTTCTTGGATTGCACATGTGTGGAGAAGATGCTGCTGAAATTGCACAG  
E T N V V L G L H M C G E D A A E I A Q -

1561 GGGTTTGCAGTTGGTATTAAAGCTGGATTAAACAAAGGCGGACTTTGATGCCACAGTAGGC  
G F A V G I K A G L T K A D F D A T V G -

1621 ATTCATCCAAGTGCAGCTGAGGAATTTGTTACCATGAGGACTCCCACTAGGAAGGTTGCA  
I H P T A A E E F V T M R T P T R K V R -

1681 AAGAACCAAGCTTCACAGGGGAAGTCAGATTCTAAAGCAAAGCTGTGGCTGGATCTTAA  
K N Q A S Q G K S D S K A K A V A G S \* -

1741 GAGTATTAATTTGCTTCAATTATTATACCCAAAGAACTTGCTGAGGCCTTAAGGCAGGT  
E Y \* F A S I I I P K E T C \* G L K A G -

1801 TATTGAGTTTTTCGAGTGATCTCTGTCAACGGAGCTTTCAAGACAATTCATGAAATAGCCT  
Y \* V F E \* S L S T E L S R Q F M K \* P -

1861 GCAGAGCTCATCTGGAAGAGGGGAGCAGTGGAATTTTGCGAGCTATTATGTGCAATTTGT  
A E L I W K R G A V E F C E L L C A I C -

1921 AATTTATTTCTCACCTTTTTTACCAATTTATTTTTCACCCTAACCTTACCCATTGTACA  
N L F L T F F T N L F F T L T L P I C T -

1981 TATTAAGATGAAATTTTCGCGAGGTACTTTGATGTTAAATAAAATAATCT  
Y \* D E I S R G T L M L N K I I -

FIGURE 3

|       |     |             |            |             |             |             |     |
|-------|-----|-------------|------------|-------------|-------------|-------------|-----|
| Peagr | 1   | APPGTERETL  | MNQAMATPLS | LSCCSPTLTR  | STLFFTKTFP  | FSSFSSTPLP  | 50  |
| Peagr | 51  | LSTKTLISLS  | PPHRTFAVRA | ESQNGADPAR  | Q...YDFDLF  | TIGAGSGGVR  | 97  |
| Psagr |     |             |            |             | MSFDFDLF    | VIGAGSGGVR  |     |
| Ecgr  |     |             |            |             | MTKHYDYI    | ATGGGSGGIA  |     |
| Humgr |     |             | MACRQ      | EPQPQGPAPA  | AGAVASYBYL  | VIGGGSGGLA  |     |
|       |     |             |            |             |             | .....       |     |
| Peagr | 98  | ASRFASNFGA  | SSAVCELPFS | TISSDTTGGV  | GGTCVIRECV  | PKKLLVYASK  | 147 |
| Psagr |     | AARFAAGFGA  | RVAVAESRY  | .....L      | GGTCVNVGCV  | PKKLLVYGAH  |     |
| Ecgr  |     | SINRAAMYGO  | KCALIEAKE  | .....L      | GGTCVNVGCV  | PKKVMWHAHQ  |     |
| Humgr |     | SARRAAELGA  | RAAVVESHK  | .....L      | GGTCVNVGCV  | PKKVMWNTAV  |     |
|       |     |             |            |             | .....       | ...         |     |
| Peagr | 148 | FSHEFEE.SN  | GFGWRYDSEP | KHDWSSLIAN  | KNAELQRLTG  | IYKNTLKNAG  | 196 |
| Psagr |     | FSEDFEQ.AR  | AYGWS.AGEA | QFDWATLIGN  | KNREIQRLNG  | IYRNLIIVNSG |     |
| Ecgr  |     | IREAIHMYGP  | DYGFD.TTIN | KFNWETLIAS  | RTAYIDRIHT  | SYENVLGKNN  |     |
| Humgr |     | HSEFMHD.HA  | DYGFP.SCEG | KFNWRIKEK   | RDAYVSRINA  | IYONNETKSH  |     |
|       |     |             |            |             | .....       | .....       |     |
| Peagr | 197 | VKLIEGRGKI  | VDA..HTVDV | DGKLYSAKHI  | LVSVGGRRPFI | P...DIPGKE  | 241 |
| Psagr |     | VTLLEGHARL  | LDA..HSVEV | DGQRFSAKHI  | LVATGGWQOV  | P...DIPGKE  |     |
| Ecgr  |     | VDVIKGFARF  | VDA..KTLEV | NGETITADHI  | LIATGGRRPSH | P...DIPGVE  |     |
| Humgr |     | IEIIRGHAAF  | TSDPKPTIEV | SGKKYTAPHI  | LIATGGMPST  | PHESQIPGAS  |     |
| Peagr | 242 | YAIDSDAALD  | LPSKBOKIAI | VGGGYIALEF  | AGIFNGLKSE  | VHVFIROKKV  | 291 |
| Psagr |     | HAITSNEAFF  | IERLPRRVLV | VGGGYIAVEF  | ASIFNGLGAE  | TTLRYRDLF   |     |
| Ecgr  |     | YGIDSDGFFA  | LPALPERVAV | VGAGYIAVEL  | AGVINGLGAK  | THLFVRKHAP  |     |
| Humgr |     | LGITSDGFFQ  | LEELPGRSVI | VGAGYIAVEM  | AGILSALGSK  | TSLMIRHDKV  |     |
|       |     |             |            |             |             | *           |     |
| Peagr | 292 | LRGFDEEIRD  | FVAENMALRG | IEFHTEESPV  | AITKAADGSL  | SLKTNKGTEE  | 341 |
| Psagr |     | LRGFDRSVRE  | HLRDELGKKG | LDLQFNSDIA  | RIDKQADGSL  | AATLKDGRVL  |     |
| Ecgr  |     | LRSEDPMISE  | TLVEVMNAEG | PQLHTNAIPK  | AVVKNTDGS   | TLELEDGRSE  |     |
| Humgr |     | LRSEDSMIST  | NCTEELNAG  | VERVLKFSQVK | E.VKKTI.SGI | FVSMVTAVPG  |     |
|       |     |             |            |             |             | *           |     |
| Peagr | 342 | .....GF     | SHIMFATGRS | PNTKDLGLES  | VGKVKAKDES  | LEVDEYSQTS  | 383 |
| Psagr |     | .....EA     | DCVFYATGRR | PMLDDLGLGN  | TAVKLTDKGF  | IAVDEHYQTS  |     |
| Ecgr  |     | .....TV     | DCLIWAIGRE | PANDNINLEA  | AGVKTNEKGY  | IVVDKYQNTN  |     |
| Humgr |     | RLPVMTMIDV  | DCLLWAIGRV | PNTKDLSLNK  | LGIQTDDKGH  | IIVDEFQNTN  |     |
| Peagr | 384 | VPSTWAIGDA  | TNRVNLTPVA | LMEGVALAKT  | LEQNEPTKP.  | DYRAIPSAVE  | 432 |
| Psagr |     | EPSILALGDV  | IGRVQLTPVA | LAEGMAVARR  | LEKPEEYRPV  | DYKLIPTAVE  |     |
| Ecgr  |     | IEGIYAVGDN  | TGAVELTPVA | VAAGRRLSER  | LFNNKPDEHL  | DYSNIPTVVE  |     |
| Humgr |     | VKGIIYAVGDV | CGKALLTPVA | IAAGRKLAAH  | LEFYKEDSKL  | DYNNIPTVVE  |     |
|       |     |             |            |             | .....       | .....       |     |

FIGURE 3 (Cont.)

|       |            |            |            |             |             |     |
|-------|------------|------------|------------|-------------|-------------|-----|
|       | 433        |            |            |             |             | 480 |
| Peagr | SQPPIGVGL  | TEEQAAEQYG | D..IDVFTAN | ERPMKATLSG  | LPDRVFMKLI  |     |
| Psagr | SLNIGTVGL  | TEEEALS..A | GHKVKIFESR | ERPMKLTLTD  | DQKTLMLV    |     |
| Ecgr  | SHPPIGTVGL | TEPQAREQYG | DDQVKVYKSS | ETAMYTAVTT  | HRQPCRMLV   |     |
| Humgr | SHPPIGTVGL | TEDEAIHKYG | IENVKTYSTS | ETPMYHAVTK  | RKTKCVMLV   |     |
|       |            |            |            | ....        |             |     |
|       | 481        |            |            |             |             | 530 |
| Peagr | VSAETNVVLG | LHMCGEDAAE | IAQGFAVGIK | AGLTRKADEDA | TVGIHPTAAE  |     |
| Psagr | VDAHDDRVLG | CHMVGAEAGE | ILOGIAVAMK | AGATKQAFDE  | TIGIHPTAAE  |     |
| Ecgr  | CVGSEEKIVS | IHGIGFGMDE | MLOGFAVALK | MGATKKDEFN  | TVAIHPTAAE  |     |
| Humgr | CANKEEKVVG | IHQGLGCDE  | MLOGFAVAVK | MGATKKADEFN | TVAIHPTISSE |     |
|       |            |            |            |             | .....       |     |
|       | 531        |            |            | 562         |             |     |
| Peagr | EFVTMRTPTR | KVRKNQASQG | KSDSKAKAVA | GS          |             |     |
| Psagr | EFVTLRTPTR |            |            |             |             |     |
| Ecgr  | EFVTMR     |            |            |             |             |     |
| Humgr | ELVTLR     |            |            |             |             |     |
|       | ....       |            |            |             |             |     |

**FIGURE 4**

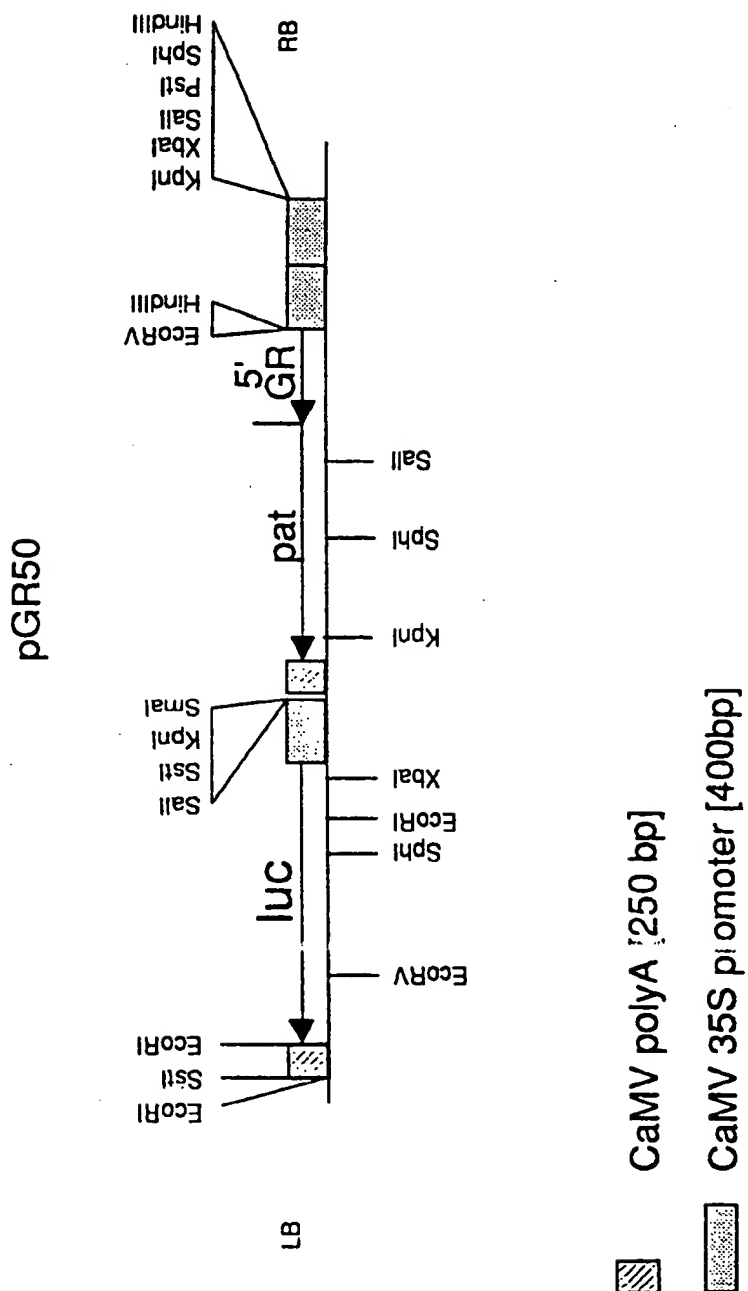
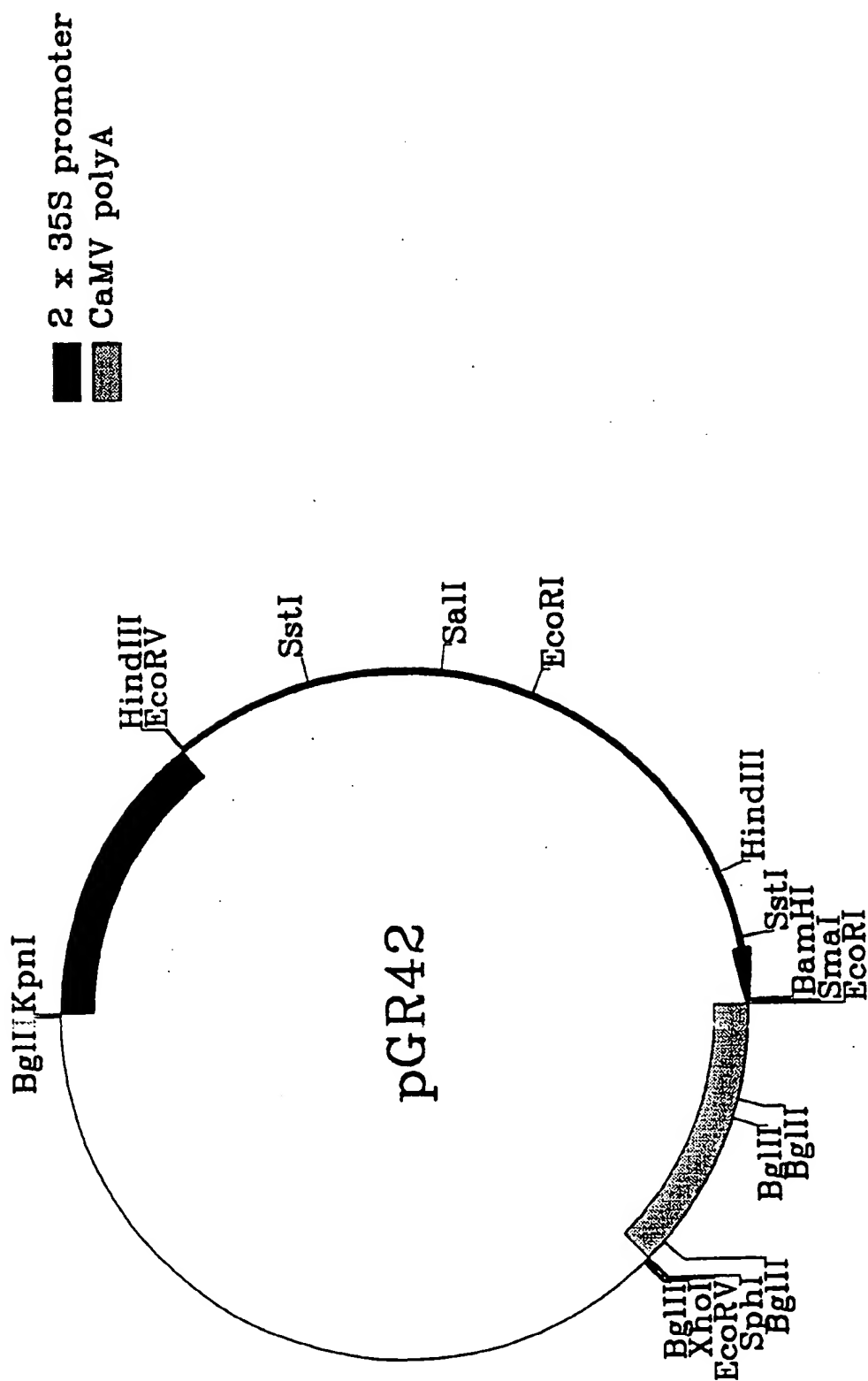




FIGURE 5



## INTERNATIONAL SEARCH REPORT

Int. onal Application No

PCT/GB 94/02058

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/53 C12N15/82 C12N15/62 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|----------|---|-----------------------|
| A        | <p>THE PLANT JOURNAL,<br/>vol.2, no.1, January 1992<br/>pages 129 - 131<br/>CREISSEN, G., ET AL. 'Molecular<br/>characterization of glutathione reductase<br/>cDNAs from pea (Pisum sativum L.)'<br/>cited in the application<br/>see the whole document<br/>---<br/>-/--</p> | 1-22                  |

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

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Date of the actual completion of the international search

2 February 1995

Date of mailing of the international search report

10.02.95

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 94/02058

## C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|------------|---|-----------------------|
| A          | <p>BIOLOGICAL ABSTRACTS, vol. 95<br/> 1 May 1993, Philadelphia, PA, US;<br/> abstract no. 101542,<br/> AONO, M., ET AL. 'Enhanced tolerance to<br/> photooxidative stress of transgenic<br/> Nicotiana tabacum with high chloroplastic<br/> glutathione reductase activity'<br/> see abstract<br/> &amp; PLANT CELL PHYSIOL.,<br/> vol.34, no.1, 1993<br/> pages 129 - 135</p> <p>---</p> | 1-22                  |
| A          | <p>THE PLANT CELL,<br/> vol.2, December 1990<br/> pages 1249 - 1260<br/> HUANG, J., 'A yeast mitochondrial leader<br/> peptide functions in vivo as a dual<br/> targeting signal for both chloroplasts and<br/> mitochondria'<br/> see the whole document</p> <p>-----</p>  | 1-22                  |